

## WHITE PAPER

# Rethinking Culture-based Microbiology – Deep Insights into any Microbiota

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- Do you need a microorganism cell bank for your natural samples that is as diverse as possible, and even includes rare species and previously uncultured species?
- Do you need microorganism cell banks from small sample volumes, restricted samples (e.g., patient biopsies) or highly dilute samples?
- Would you like to add sample-specific growth stimulators such as tissue fluids from biopsies or gut lavage, but you also deal with limited samples?
- Are you looking for an efficient way to singularize cells of complex communities and obtain guaranteed monoclonal colonies in standard lab formats such as multi-well plates with minimal turnaround time and dramatically reduced consumption?

**Key words:** cultivation of uncultured; droplet microfluidics; cell bank service

## Introduction

If you answered yes to one of these questions, Biomillenia is your partner (Table 1). We recognized and seized the potential of picoliter-sized droplets as miniaturized bioreactors for highly parallelized cultivation of bacteria early on, and built our proprietary platform for droplet microfluidics accordingly. Following the rationale of dilution to extinction, we compartmentalize single bacteria in droplets and provide them with their own space and nutrient stock for growth, thereby preventing competition. In contrast to standard dilution to extinction approaches in which a well of a microtiter plate (MTP), for instance, is equivalent to one of our droplets, pL-droplets offer more suitable volumes for replication of single cells, because important growth effectors like signaling molecules can reach physiological concentrations in the miniaturized compartments. At the same time, the small size of our droplets

allows us to produce billions of droplets at high speed, meaning we reach an unprecedented sampling depth for your microbial sample. The species diversity of your cell bank profits from our high-throughput approach as this increases the chance of finding and culturing rare cell types.

Simultaneously, you benefit from reduced consumption and time due to the concept of miniaturization. In MTPs, you would need 2000 L of medium to fill  $10^7$  wells with 200  $\mu$ L for single-cell culturing experiments, while we need just 0.2 mL medium in total for the same number of 20 pL droplets. This is a 10 million-fold reduction alone in medium consumption, without even taking the minimal use of plastic consumables in our experiments into account. Assuming you have specialized liquid-handling robots that can fill one well per second, you would need 116 days to fill all  $10^7$  wells. We speed up this process one thousand-fold and offer you  $10^7$  droplets in 2.8 hours, as

### Introduction to droplet microfluidics - Droplet microfluidics is a superior alternative to MTP wells

Droplet microfluidics is a technique that utilizes 2 immiscible phases: a continuous flowing oil phase and a dispersed aqueous phase. With the help of the oil phase, small aqueous droplets are generated at high speed in micrometer-channel devices. A biocompatible surfactant dissolved in the oil forms an amphiphilic monolayer at the interface of the water and oil, thereby stabilizing the droplets. Hence, droplets are small, stable vessels in which experiments can be conducted. With Biomillenia's proprietary microbiome-on-a-chip technology, platform droplets can be created on average at speeds of 10,000 droplets/s. Droplet volumes are precisely adjusted in a range down to a few picoliters and up to a few nanoliters, depending on the experimental requirements. Due to the dramatic miniaturization, droplet populations easily comprising 100 million individual droplets are processed within hours, at the same time keeping the consumption of valuable reagents to a minimum (Figure 1).

**Table 1. Partnering with Biomillenia for typical commercial applications**

|                       |                     |  |
|-----------------------|---------------------|--|
| <b>Human health</b>   | Pharmaceuticals     | Microbiome-derived compounds<br>Compounds targeting microbiome<br>Effect of microbiome on drug utilization |
|                       | Food Supplements    | Probiotics / Prebiotics  |
|                       | Personal Care       | Microbiome-friendly skin care  |
| <b>Agriculture</b>    | Natural fertilizers | Bacterial strains converting N <sub>2</sub> , phosphate  |
|                       | Biopesticides       | Natural fungicides, insecticides   |
|                       | Animal health       | Improved feedstock conversion, antibiotics replacement, non-GMO  |
| <b>Sustainability</b> | Biofuels            | Improved non-GMO fermentation strains  |
|                       | Enzymes             | Directed evolution for fit-for-purpose enzymes   |
|                       | Green Chemicals     | Strain optimization for compound production  |

we generate droplets at 1000 Hz.

Interested in our technology? Continue to read an introduction to droplet microfluidics on page 2.

### Create rich and diverse cell banks containing rare and even previously uncultured species

With our expertise in microfluidic droplet handling, we can incubate droplets under controlled conditions for up to several months, if required, without having to consider the common problems of microscale liquid handling such as evaporation or cross-contamination. Thereby, we obtain millions of monoclonal cell cultures in droplets that can be transferred by our advanced droplet positioning strategies to larger liquid volumes. We use these techniques as an interphase to standard lab formats such as MTP, enabling our customers to fully benefit from the advantages of droplet microfluidics without having to adapt

**Table 2. Microfluidic droplets allow a 10 million-fold reduction in medium consumption and one thousand-fold higher throughput than standard cultivation formats for highly parallelized cultivation.** The starting value of 10<sup>7</sup> cultivation compartments is compared for standard 96-well plates and microfluidic droplets in terms of medium and time requirements.

|                     | MTP             | Droplets        |
|---------------------|-----------------|-----------------|
| No. compartments    | 10 <sup>7</sup> | 10 <sup>7</sup> |
| Volume/compartiment | 200 µL          | 20 pL           |
| Total volume        | 2000 L          | 0.2 mL          |
| Throughput          | 1/s             | 1000/s          |
| Total time          | 116 d           | 2.8 h           |

their own lab processes to this.

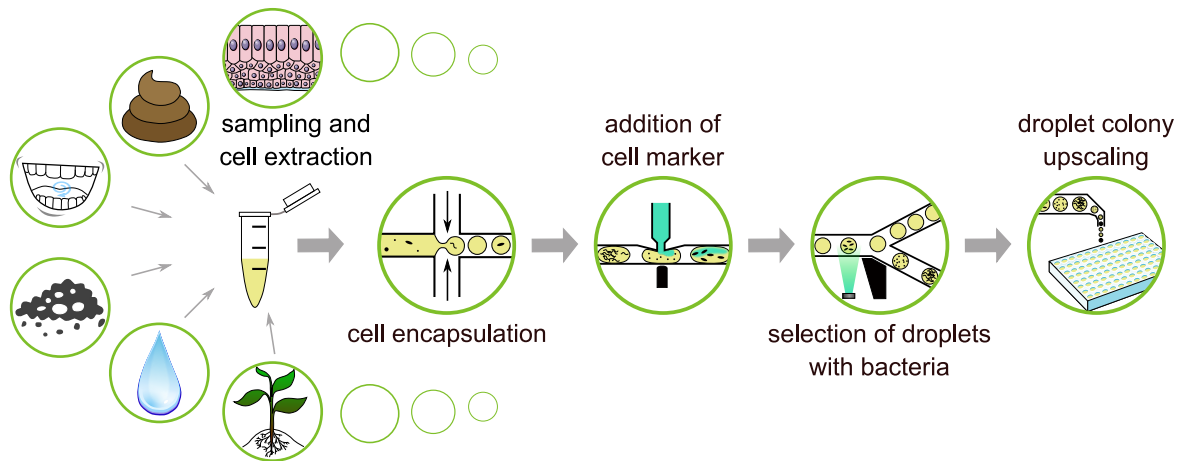
You can decide whether all droplets containing cells should be exported from our platform or you can also choose a further screening step to only export cells with a specific function. Whatever decision you make, you will profit from our high throughput and extraordinary sampling depth, resulting in comprehensive cell banks with rare species and even previously uncultured species.

Here, we showcase the increased microbial diversity achieved with droplet-based cultivation starting from human oral cavity samples. We give a step-by-step overview of the experimental workflow, in which we compare the cultivation outcome in droplets with that obtained by agar plate cultivation. To highlight the experimental range of our technology we also include regulation of nutrient and oxygen availability.

### Step-by-step overview of the droplet-based workflow

Using the oral microbiome as a model habitat, we demonstrate a droplet-based workflow for the cultivation of novel species on our microfluidic platform. To illustrate the advantages of highly parallelized single-cell encapsulation and incubation in pL-droplets, we compare the cultivation outcome obtained with in-droplet cultivation to standard agar plate cultivation.

The workflow consists of 5 main steps (Figure 2). First, the oral microbiome is sampled and the bacterial fraction is extracted. Then, droplets and plates are inoculated with the complex cell mix in two different media and under oxic and anoxic conditions and incubated in the third



**Figure 1. Microfluidic droplets are a competitive alternative to MTP wells enabling one millionfold reduction in volume consumption.** Our experts in microbiology extract the microbial community from a variety of your samples, which could be derived from humans or diverse environmental habitats. Single cells are encapsulated in droplets, together with chemically defined nutrients and/or a cell-free extract of the sample environment. The cells are incubated in droplets for days to months under specially adapted conditions. In the following step, we can add a cell marker or other substrates to droplets to select only droplets with cells or only droplets containing cells with a specific functionality. In the last step, we deposit droplets with cells in standard lab formats to create your diverse culture collection of monoclonal strains.

step. After this, the cells from one cultivation technique are pooled and used for subsequent DNA extraction and amplicon preparation. The final step is to determine the microbial diversity achieved with the different cultivation formats by preparing and sequencing the 16S rRNA amplicon.

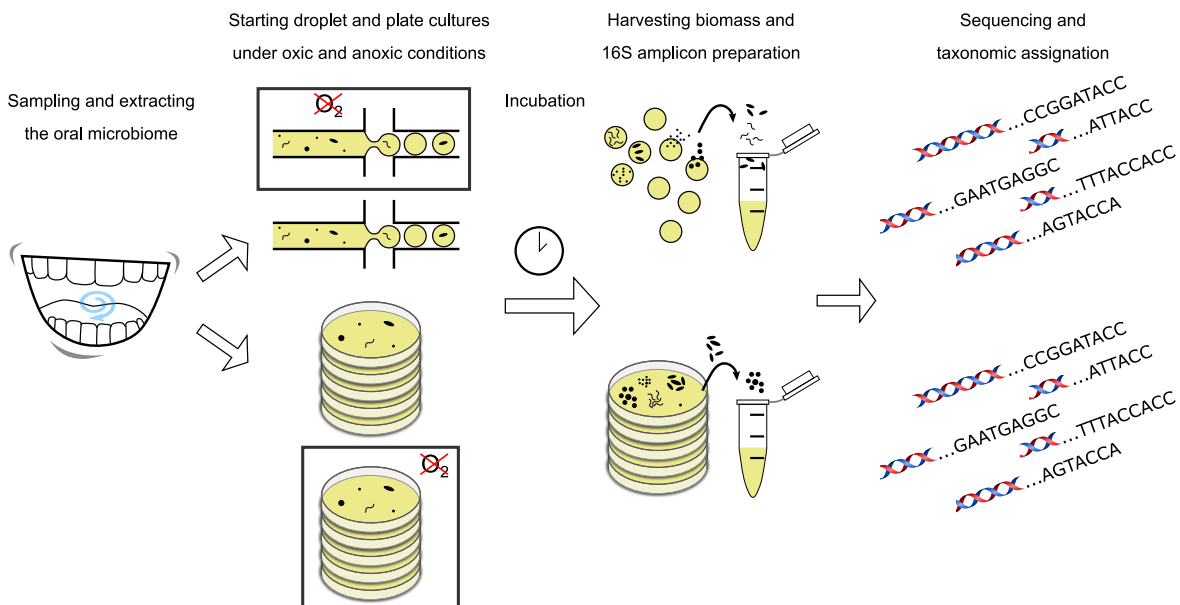
### From the microbial complexity of the oral cavity to a bacterial cell bank

The oral microbiome is an ideal source of complex natural microbial communities, but any other type of starting material including tissue, soil, feces, water etc., could be

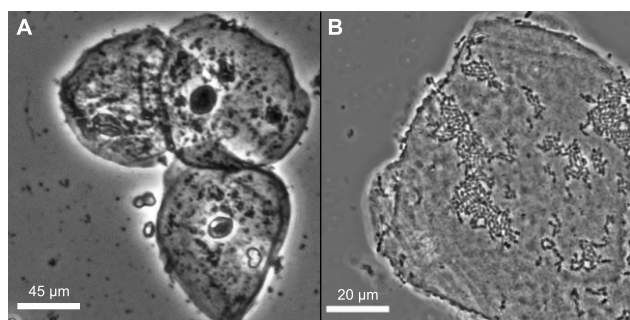
used. Microscale cultivation systems such as our droplets are especially convenient when dealing with low-volume samples or samples with highly restricted access such as patient biopsy samples.

We sampled the oral microbiome of 7 healthy volunteers by flushing their oral cavity with mineral water for 20–30 seconds and collected the liquid in sterile vessels. The 7 samples were subsequently pooled and immediately processed.

We designed the extraction procedure to separate the bacterial fraction including bacteria and archaea from the sample without impairing their viability or biasing the community structure towards specific genera. These pro-



**Figure 2. The sampling depth achieved with droplets is superior to that from agar plates.** The workflow for comparing the cultivation outcome of in-droplet cultivation with agar plate cultivation is shown. The oral microbiome is sampled and the cell suspension extracted from the mouth flush sample is used to inoculate pL-droplets and agar plates of the same medium. For both cultivation formats incubation under oxic and anoxic conditions is started. One droplet population comprises several billion droplets and will be compared later to a plate stack of 5 plates. For each condition 5 replicates are prepared. Cells are incubated for 7 days under the respective cultivation conditions. The bacterial biomass is collected from droplet populations and plate stacks. All cells growing in one droplet population are pooled and represent the cultivation outcome for this droplet population. All cells growing on one plate stack are pooled and represent the cultivation outcome for this plate stack. DNA is extracted and amplicons of the 16S rRNA gene are prepared for each cell pool. To determine the community structure and diversity for the cultivation outcome of in droplet and plate cultivation, the 16S amplicons are sequenced and taxonomy is assigned.



**Figure 3.** In mouth flush samples, bacteria adhere closely to eukaryotic epithelial cells. Microscopic images of an oral microbiome sample. Images were recorded in phase contrast. A) Eukaryotic epithelial cells in close contact. B) Rod-shaped bacteria attached to a eukaryotic cell.

protocols usually undergo extensive optimization cycles, taking the unique sample characteristics for the chosen sample type into account. Besides extracting bacteria, we can also target other cell types such as fungi for downstream applications in droplets.

We needed a strategy for the oral microbiome to detach and separate the bacterial cells from eukaryotic epithelial cells, which were abundant in the sample and often appeared in close contact with each other and bacterial cells (Figure 3). Hence, we homogenized the sample using shear forces and separated the treated cell suspension by size. We determined the concentration of viable cells in the resulting bacterial fraction using live/dead staining.

### Microfluidic approach enables cultivation of 1000-fold more cells

The concentration of viable bacterial cells is important during the subsequent single-cell encapsulation in droplets. Because the probability of droplets containing one or multiple cells follows the Poisson distribution, we adjusted the concentration of viable cells to 0.5 cells per droplet on average, which translates to 0.5 cells/20 pL in this experiment. Thereby, we can ensure that the majority of droplets containing cells are occupied by only one cell (75% of occupied droplets). At the same time 60% of all droplets remain empty, which is a trade-off for the low number of droplets containing several cells.

Due to our high-throughput droplet generation (more than 8000 droplets per second) and the small volume of our droplets (20 pL), we generated and incubated droplet populations comprising  $2.5 \times 10^7$  droplets, which amount to only 500  $\mu\text{L}$  in total volume. This means we encapsulated  $1.25 \times 10^7$  cells in droplets reaching an extraordinary sampling depth for the oral microbiome. In comparison, on a plate stack (5 plates of the same medium), which we define as equivalent to a droplet population in this experiment, we could inoculate only  $3.75 \times 10^4$  cells in total to obtain colonies on plates that were still distinguishable. The advantage of microscale cultivation in droplets becomes apparent, enabling sampling of 1000-fold more cells and thereby increasing the chance of cultivating rare cell types and finding metabolically active cells among dormant cell populations.

We used a medium with high concentrations of mucin to replicate the original environmental conditions as closely as possible. The mucin-containing medium, characterized by higher viscosity, is a good example of our ex-

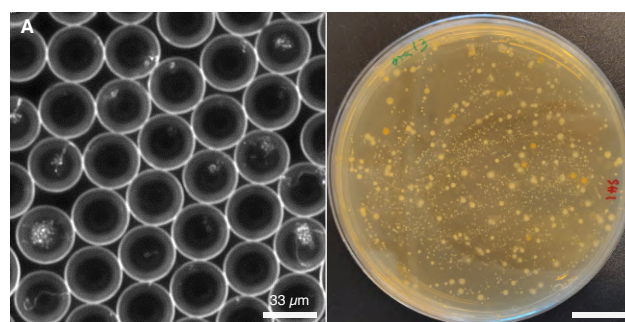
perience in using a broad range of media in the microfluidic system. We also incubated the oral microbiota in fundamentally different media and recommend doing this to achieve highest species diversity during creation of cell banks (data not shown). To demonstrate the versatility of possible incubation conditions in addition to different media, we included oxic and anoxic incubation conditions in the study setup. For each of the 4 combinations of medium, oxygen availability and cultivation format, we prepared five replicates amounting to 10 droplet populations and 10 plate stacks in total. These were incubated for 7 days at 30°C. The incubation parameters including temperature, atmospheric conditions and incubation length can be easily adapted within a broad range to the specific needs of the selected sample material.

At the end of the cultivation period, microbial growth is visible in droplets as well as on plates (Figure 4). We extracted the microbial biomass from the droplets and pooled it for each droplet population. A comparable procedure was carried out for the agar plates, consisting of washing the bacterial colonies and pooling the biomass for the five plates comprising one plate stack.

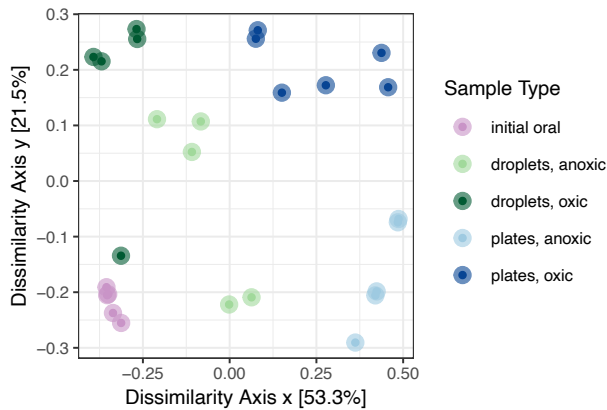
The biomass pools, which represent the cultivation outcome achieved with either droplets or plates, and samples of the initially extracted oral microbiome were subjected to DNA extraction. We prepared amplicons of the full 16S rRNA gene from the metagenomic DNA, which we sequenced along with amplicons of a mock community. We assigned taxonomy to the quality processed reads to determine the community structure and species diversity obtained with both cultivation methods.

## Results

We performed an unconstrained ordination to investigate the general differences between the community composition of the different samples (Figure 5). By looking at the spread of the data we can determine how reproducible the bacterial community is that we achieved with the different cultivation formats and which sample types are more similar than others. First, the replicates for one sample type, depicted by dots of the same color, cluster together, meaning we achieved reproducible cultivation outcomes in our study. Second, there is a clear difference between the community grown in droplets (green dots) and the community on plates (blue dots). The collection of species that grew in droplets resembles the initial community derived from the mouth flush samples (pur-



**Figure 4.** Detection of small or low-density cultures is easier in droplets. Microbial growth in droplets and on plates after 7 days of incubation. A) Microscopic image with darkfield illumination of droplets. B) Picture of agar plate.



**Figure 5. Droplet samples are more similar to initial oral samples than plate samples.** The difference in community structure caused by different cultivation conditions. A principal coordinate analysis based on the Bray-Curtis distance matrix is shown.

ple dots) more closely than the species growing on plates. Finally, within the two different cultivation methods we observe a stronger effect by using different media than by exposing the samples to different oxygen availabilities (data not shown).

We plot the community structure at the genus to learn more about which groups of bacteria grew within droplets or plates (Figure 6). As expected, there are pronounced differences in the abundance of genera between communities obtained during in-droplet and agar plate cultivation. While the most abundant genus in droplet samples is *Neisseria*, which is consistent with the initial oral community, in plate samples the genus *Streptococcus* is strongly overrepresented in the community. As the abundance of *Streptococcus* is much lower in the initial community, its growth seems to be favored during cultivation on agar plates, at the cost of the growth of other species. Also, in droplets we find a higher abundance of *Streptococcus*. However, this genus does not dominate the entire community, most likely due to cell encapsulation, which prevents overgrowth of fast-growing species in droplets. The third most abundant genus in the initial community is *Fusobacterium*. This genus is only represented in relevant amounts in the oxic droplet samples, showing the capacity of droplets to establish a favorable growth environment for a wide variety of species.

To summarize the comparison of droplet and agar plate cultivation, we computed alpha indices (Figure 7).  $\text{Chao}_1$ , an index for species richness, and the Fisher index for evenness are for both droplet-plate comparisons higher for droplets than the corresponding plate sample. This is another indication that the recovered microbial diversity of growing strains is larger and resembles the initial microbial diversity more closely when in-droplet cultivation is used.

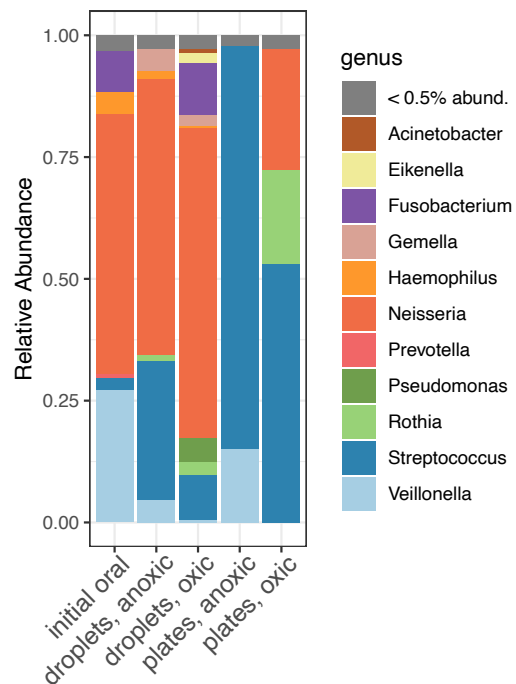
## Conclusion

We demonstrated the advantages of Biomillenia's proprietary microbiome-on-a-chip technology compared to standard microbiological cultivation methods. With in-droplet cultivation we obtained a higher species richness and evenness among the bacteria that were able to grow. In particular, the overgrowth of *Streptococcus* species, as

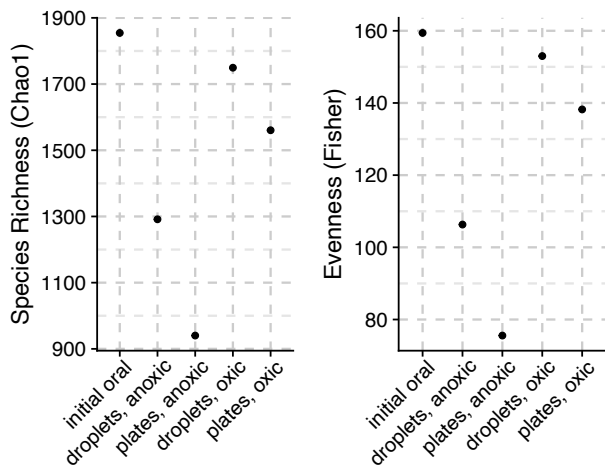
observed on agar plates, was prevented in droplets because the competition for nutrients and space between cells is intercepted by single-cell encapsulation. Besides the larger number of recovered species, the community composition after cultivation in droplets resembles the initial composition more closely, hence subsequent cell banks will be more representative for the chosen sample material.

## On-demand workflows tailored to our customers' needs

While the presented results were obtained for the oral microbiome derived from humans, virtually any other sample material could be used. It is possible to process, in particular, low-volume samples or samples subject to restricted access with our technology, due to our small sample consumption. As our team includes many expert microbiologists, we can extract microbial fractions suitable for microfluidics from any kind of sample, regardless of how challenging the sample is. Our microfluidics engineering expertise complements our microbiological expertise and allows us to monitor and regulate various experimental parameters during droplet experiments. In addition to fully anoxic or oxic workflows, we can also realize other critical conditions such as pH shifts, defined temperature profiles or a combination of these. To reach the highest possible species richness in the cell banks, we also use the droplet-inherent high throughput to cultivate in a range of different media, which can also contain lipophilic or particulate substances. With our proprietary development, we can parallelize the droplet generation of up to 60 different media.



**Figure 6. The community structure of droplet samples resembles more closely the community found in initial oral samples than plate samples.** Community structure displayed at the genus level for all sample types. Color-coded bars of the stacked bar chart display the relative abundance of genera with an abundance above 0.5%.



**Figure 7. Diversity indices are higher for anoxic and oxic incubated droplet samples than for the corresponding plate samples.** Comparison of alpha diversity indices for all sample types.

As our microfluidic system is modular, we create on-demand workflows tailored to the scientific question of customers and it can include targeted selection of specific cell variants or droplet transfer to standard formats. For design of the required screening assays, a multitude of read-out techniques compatible with high throughput are available. From multicolor fluorescence-based read-outs, through label-free image-based selection and next-generation sequencing, we can realize a variety of experiments in microscale format.

We provide our customers with comprehensive culture collections with rare and even previously uncultured bacteria among the isolates, which are delivered in standard lab formats for rapid integration into their own processes.

## Disclaimer

For information on partnering with Biomillenia, go to <https://biomillenia.com/develop-innovative-bacterial-products-with-biomillenia/>

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